

[54] PHOSPHOLIPASE A2 INHIBITOR

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514/928

[58] Field of Search 260/403, 928; 558/160;
514/102, 103

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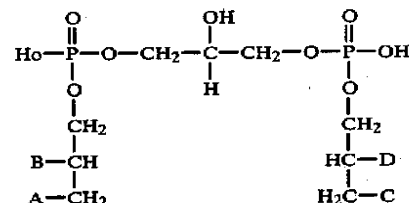
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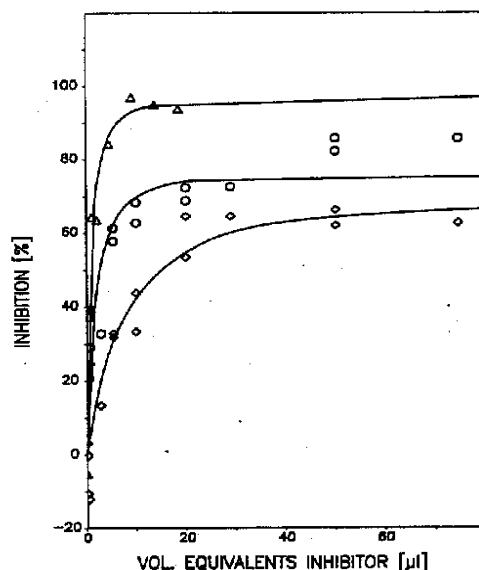
[57] ABSTRACT

Potent, specific inhibitors of phospholipase A₂ are dis-
closed which are compounds of the formula:



wherein A is R₁CO₂ or OH, B is R₂CO₂ or OH, D is
R₃CO₂ or OH and C is R₄CO₂; and wherein R₁, R₂, and
R₃ and R₄ are each C₁₀-C₃₀ alkyl groups comprising
from about 0-3 double bonds.

8 Claims, 4 Drawing Sheets



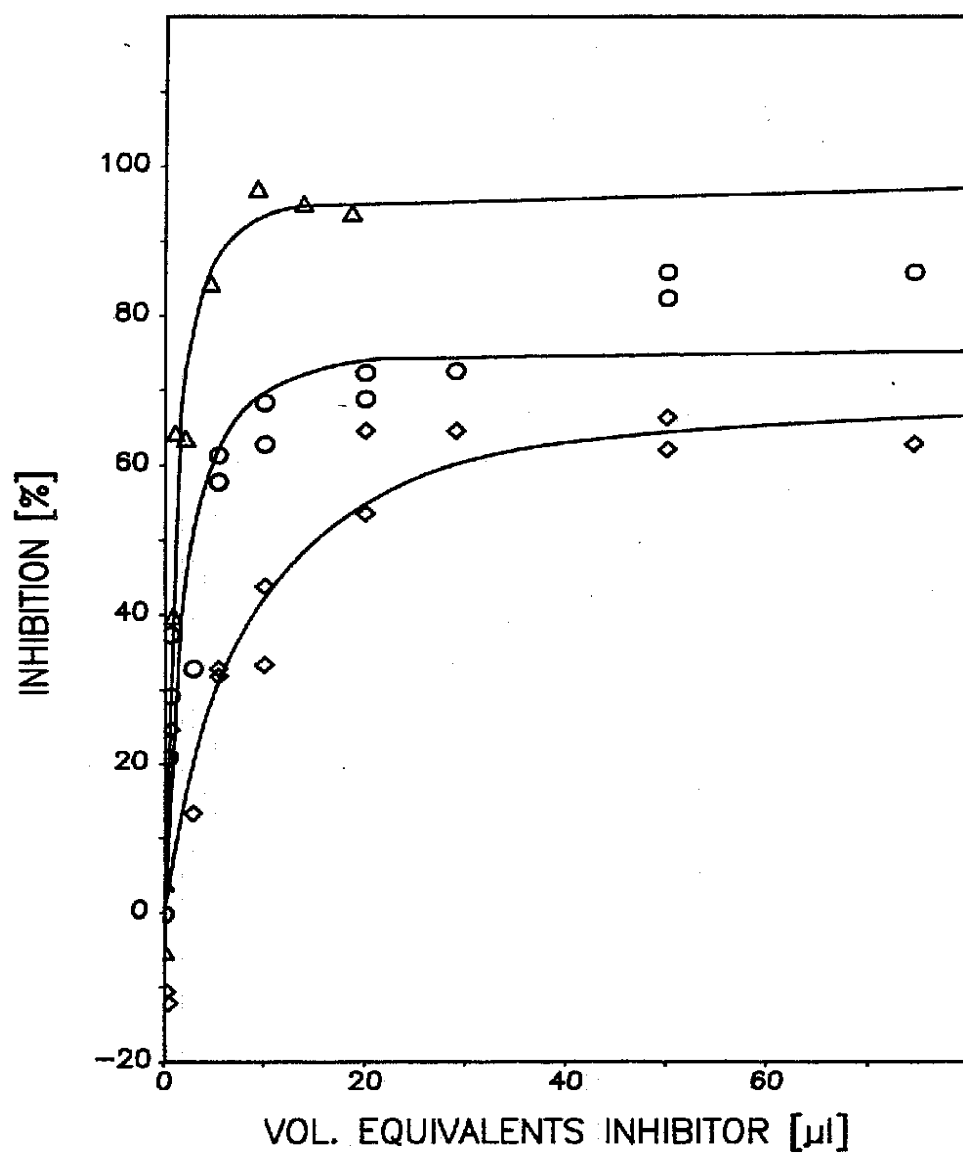


FIG. 1

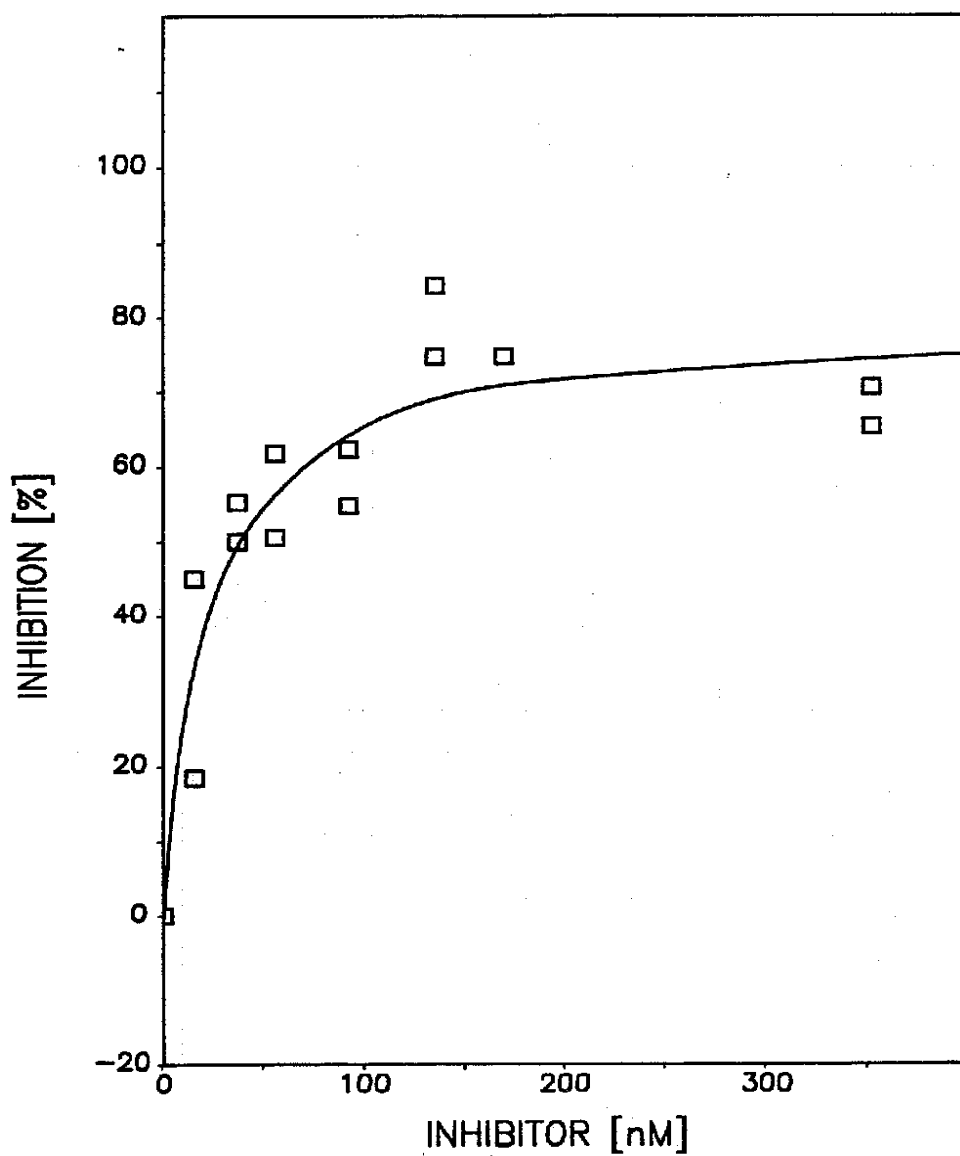


FIG. 2

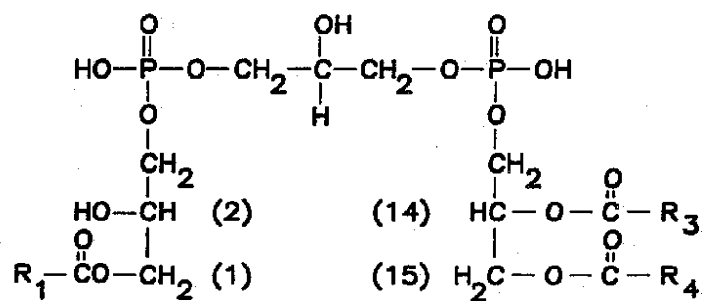


FIG. 3

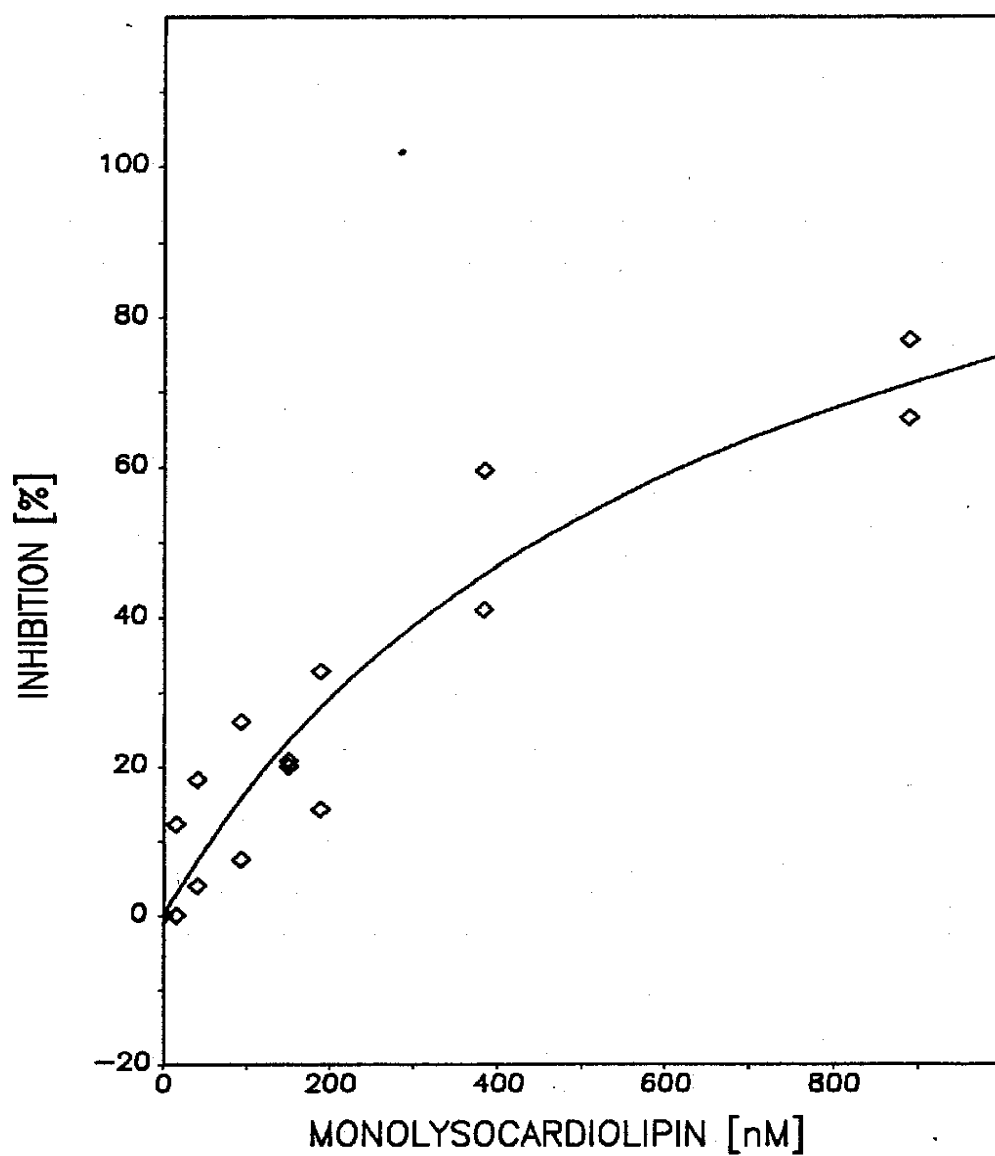


FIG. 4

PHOSPHOLIPASE A₂ INHIBITOR

This invention was made with the assistance of Grant No. HL08214, awarded by the National Institute of Health, and the Hormel Foundation. The Government has certain rights invention.

BACKGROUND OF THE INVENTION

Mitochondria from many sources possess phospholipase A₂ activity associated with both their inner and outer membranes. The physiological functions of this enzyme or enzyme group has not been established with certainty although several reasonable, potential functions can be hypothesized. These include the possible involvement of mitochondrial phospholipase A₂ in regulating membrane permeability and therefore such processes as calcium ion (Ca²⁺) accumulation and release, the efficiency of ATP synthesis, and cellular thermogenesis. Other possible functions of this enzyme include the modification of phospholipid acyl group composition, the initiation phospholipid degradation, and the liberation arachidonic acid to initiate the "arachidonic acid cascade," which involves the synthesis of prostaglandins and related compounds. While generally the products of the cascade are beneficial, in certain disease processes and other conditions the production of prostaglandins and similar products induces deleterious consequences such as inflammation (see paper by N. A. Plummer et al., abstracted in *Journal of Investigative Dermatology*, Vol. 68, No. 4, p. 246 (1977)); erythema; platelet aggregation (B. B. Vargaftig, *J. Pharm. Pharmacol.*, Vol. 29, p. 222-228 (1977)); and the release of SRS-A (slow reacting substance-anaphylaxis), a known mediator of allergic responses. The inhibition of phospholipase A₂ can prevent these and similar conditions mediated by the action of this enzyme.

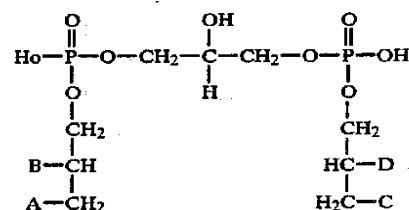
In cells which are injured by ischemia, chemical, mechanical or other means, the mitochondria develop impaired functional capacities and the extent of these metabolic lesions can be pivotal factors in the recovery or death of the injured cell. Persistent activation of mitochondrial phospholipase A₂, arising because of loss of cellular Ca²⁺ homeostasis, is a probable dominant mechanism by which mitochondrial damage develops. There is also reason to suspect that phospholipase A₂-dependent loss of mitochondrial function may play an important role in the etiology of several significant human diseases including Reye's syndrome, muscle wasting diseases and malignant hyperthermia.

Thus a need exists for compounds which act to inhibit the activity of phospholipase A₂. Useful inhibitory compounds would be specific, potent and nontoxic. The inhibitors could be employed to control mitochondrial damage and damage to other membranous structures in injured or diseased mammalian tissue and thereby limit permanent damage and improve cellular survival. Hereinafter the deleterious effects and pathologies dependent entirely or in part upon phospholipase A₂ activity will be referred to as phospholipase A₂ mediated conditions (PMC).

SUMMARY OF THE INVENTION

The present inventions are directed to compounds which are highly active to inhibit phospholipase A in vivo or in vitro. Thus, such compounds would be expected to generally be useful to treat or prevent PMC in mammals, e.g. domesticated animals and humans. The

compounds of the present invention can be generally represented by formula I:



wherein A is R₁CO₂ or OH, B is R₂CO₂ or OH D is R₃CO₂ or OH and C is R₄CO₂; wherein each of R₁, R₂, R₃ and R₄ is a C₁₀-C₃₀ alkyl group, preferably a C₁₂-C₂₂ n-alkyl group comprising from 0-3 double bonds.

Therefore, the A-D acyl groups, are preferably derived from saturated or unsaturated fatty acids such as an alkanolic, alkenolic, dienolic or trienolic acids. Such acids include lauric, oleic, palmitic, linoleic, linolenic, stearic, eleostearic, palmitic, palmitoleic, petroselinic, vaccenic, erucic acid and the like and mixtures thereof. Preferably, one of A or B is OH, or B and D are OH.

Formally, the compounds of the present invention are the partial hydrolysis products of a known, naturally occurring compound, cardiolipin. In its "pure" form, cardiolipin can be represented by formula I wherein A, B, C and D are each RCO₂ moieties, wherein RCO₂ is derived from a variety of saturated and unsaturated fatty acids, such as those described hereinabove. The preferred compositions of the present invention can be isolated from mammalian mitochondria, and so are probably derived from in vivo hydrolysis of cardiolipin. For example, the compound designated as monolysocardiolipin can be represented by formula I wherein A is R₁CO₂, C is R₄CO₂, D is R₃CO₂, and B is OH. Iso-monolysocardiolipin can be represented by formula I wherein B is R₂CO₂, C is R₄CO₂, D is R₃CO₂ and A is OH, while in dilyocardiolipin A is R₁CO₂, D is R₃CO₂ and B and C are OH.

Although small amounts of certain of these compounds have been formed by hydrolysis of cardiolipin (*J. Biol. Chem.*, 249, 3423 (1974)), the present invention is directed to pharmaceutically effective dosage forms containing mono- and dilyocardiolipin and the analogs thereof within the scope of formula I, in substantially pure form. Therefore, the present invention is directed to phospholipase A₂ inhibitors which can be structurally defined with precision, e.g., wherein the structure of each acyl (RCO₂) group is known; and to phospholipase A₂ inhibitors wherein the RCO₂ groups are varied to the extent that the active composition is a mixture of compounds falling within the scope of formula I.

For example, the composition designated monolysocardiolipin is made up of compounds of formula I which yield at least twelve different fatty acid residues upon hydrolysis (see Table II, below). On the other hand, mono- or dilyocardiolipins of homogeneous composition may be readily prepared by selectively protecting, acylating and deprotecting the five hydroxyl moieties present on the compound of formula I wherein A=B=C=D= hydroxy. Methods for the selective preparation of esters and for the selective protection and deprotection of OH groups are well-known in the art. See, e.g., *Compendium of Organic Synthetic Meth-*

ods, I. T. Harrison et al., eds., Wiley-Interscience, N.Y. (1971) and J. Org. Chem. 37 1947 (1972).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graphic depiction of the recovery of phospholipase A₂ inhibitor activity in three fractions from the procedure employed to isolate one of the inhibitors of the present invention;

FIG. 2 is a graphic depiction of the inhibition of phospholipase A₂ activity by monolysocardiolipin derived from rat liver;

FIG. 3 depicts the structural formula of monolysocardiolipin;

FIG. 4 is a graphic depiction of the inhibition of phospholipase A₂ activity by monolysocardiolipin isolated from bovine heart.

DETAILED DESCRIPTION OF THE INVENTION

During the purification of phospholipase A₂ from rat liver mitochondria, as described by Aarsman et al. in Biochem. Biophys. Acta., 792, 363 (1984), solubilization of the enzyme by production of an acetone powder extract was found to yield only about five percent of the starting enzyme activity. However, subjecting the extract to gel filtration chromatography increased the recovered activity by about ten-fold, as summarized on Table I, below. These findings suggested to us that an inhibitor, which was removed by gel filtration, was present in the acetone powder extract.

TABLE I

PHOSPHOLIPASE A₂ PURIFICATION SUMMARY

Fraction	Protein Recovery mg	Activity Recovery nmol/min	Specific Activity mU/mg	Fold Purification
Mitochondria	1699.0	1607	1.0	1
Acetone Powder Extract	1310.0	78	0.1	0.1
AcA54 Chromatography Fractions	1.8	880	767	808
Ultrafiltrate	1.7	234	136	144
Dialysate	1.6	165	109	114
Affinity Column				
Frac. 22-28	0.03	131	4503	4740
Frac. 23	0.01	39	7923	8340

Examination of gel filtration column fractions revealed phospholipase A₂ inhibitor activity eluting to the void volume. This was a turbid fraction containing both lipids and protein. The inhibitor activity partitioned to the organic layer of Folch extracts and was thus presumed to be a lipid. When these extracts were subjected to thin layer chromatography (TLC), more than one spot was found to contain inhibitor activity. In addition, all active spots contained phosphorous, suggesting that the inhibitor(s) was a phospholipid.

FIG. 1 illustrates the recovery of inhibitor activity through these steps of purification. The fractions were assayed with respect to their ability to inhibit mitochondrial phospholipase A₂ activity essentially by the procedure of Aarsman et al., in Biochem. Biophys. Acta., 792, 363 (1984), the disclosure of which is incorporated by reference herein. The media contained Triton X-100 (octoxynol-9) at a 1:1 mole ratio with respect to the substrate phosphatidylethanolamine. Triangular symbols (Δ) show the activity of void volume column frac-

tions from the gel filtration column. Circular symbols (○) show the recovery of this activity in the CHCl₃ layer of a Folch extract. Finally, the diamond symbols (◇) show inhibitor activity eluted from a discrete spot on TLC plates on which the lipid components in the Folch extract had been separated. The spot utilized was located between phosphatidylethanolamine and cardiolipin spots when plates were prepared with silica gel H and developed with chloroform:methanol:water (65:25:4). This inhibitory component was selected for structural elucidation because its activity was higher than other components on a per lipid phosphorous basis. FIG. 2 shows the inhibitory activity of the purified component expressed on the phosphorous basis.

Subjecting the purified component to basic hydrolysis yielded phosphate, glycerol and free fatty acids in ratios of 1:1.3:1.3, respectively, on a molar basis. The composition of fatty acids obtained was determined and is summarized in Table II.

TABLE II

FATTY ACID COMPOSITION OF THE PURIFIED INHIBITORY COMPONENT

Fatty Acid ^a	Weight ^b Percentage
Palmitic acid	3.71
Stearic acid	1.69
Oleic acid	3.75
n.i.	6.23
n.i.	4.24
Linoleic acid (18:2ω6)	61.99
n.i.	2.54
Linolenic acid (18:3ω3)	4.27
n.i.	3.57
n.i.	3.35
Ecosadienoic acid (20:2)	2.14
Ecosatrienoic acid (20:3)	2.52

^an.i.: not identified.

^b17:0 (heptadecanoic acid) was used as internal standard. Fatty acids isolated during the basic hydrolysis of the purified inhibitory component were converted to methyl esters and quantitated by standard GLC procedures.

The ratios of the three molecular constituents, together with the prevalence of linoleic acid (18:2) among the fatty acids indicated that the inhibitor is monolysocardiolipin. The structure of this compound is depicted in FIG. 3, wherein R₁CO₂, R₃CO₂ and R₄CO₂ are primarily 9, 12-octadecadienoyl residues. IR spectra of the purified inhibitor gave further evidence for this structure since they were similar to the spectrum of cardiolipin, except that the unsymmetrical band of the phosphate and hydroxyl groups at 3200 cm⁻¹ appeared as a symmetrical band at 3380 cm⁻¹. This alteration can be taken as an indication of the additional hydroxyl present in monolysocardiolipin.

To confirm the structure of the present inhibitor, monolysocardiolipin was prepared from bovine heart cardiolipin by treatment with snake venom (*Crotalus adamanteus*) phospholipase A₂, essentially as described in Eichberg, in J. Biol. Chem. 249, 3423 (1974), the disclosure of which is incorporated by reference herein. This material and the inhibitor purified from the acetone powder extract showed identical TLC migration behavior using several solvent systems.

FIG. 4 shows that the monolysocardiolipin derived from bovine heart is a potent inhibitor of the mitochondrial phospholipase A₂.

As described hereinabove, TLC plates obtained from Folch extracts of inhibitor containing column fractions indicated the presence of more than one active species. One additional active fraction was less polar while the other was more polar than monolysocardiophilin, based on the migration behaviors observed. After identifying monolysocardiophilin, it was confirmed that the parent lipid, cardiophilin, was the less polar species while dilysocardiophilin compound was the more polar one. In FIG. 3, replacement of the R_3CO_2 moiety by OH would yield the structural formula of dilysocardiophilin. These inferences were confirmed by comparing the properties of two known samples of these compounds to those of the two additional inhibitory components obtained in the Folch extracts. It is probable that cardiophilin derives a substantial portion of its inhibitory activity extent by serving as a substrate for mitochondrial phospholipase A_2 and thereby resulting in production of the more potent mono and dilyso degradation products.

Properties of monolysocardiophilin relevant to its use as an inhibitor of phospholipase A_2 and therefore as a potential therapeutic agent and research tool, can be summarized as follows:

(1) The inhibitor is very potent in that 1 part in approximately 3000 (molar basis with respect to substrate) reduces mitochondrial phospholipase A_2 activity by 50%.

(2) The inhibitor is likely to function by a direct but noncovalent interaction with the enzyme. A direct mechanism is indicated by activity *in vitro* against the purified enzyme. Noncovalent interaction is indicated by loss of inhibition upon gel filtration. Absence of inhibition through an effect on the substrate's physical state is indicated by the low level at which monolysocardiophilin is active with respect to the substrate level.

(3) The high potency suggests potential high specificity and low toxicity were the agent to be used as an inhibitor *in vivo*.

(4) The inhibitor is a naturally occurring compound which would be subject to removal by both anabolic (reacylation) and catabolic (further hydrolysis) metabolism. No significant toxicity would exist in the products of either of these potential pathways for inhibitor metabolism.

(5) The potency of the inhibitor is expected to be adjustable by altering the position of the acyl group chains. This is indicated by the fact that monolysocardiophilin obtained from liver is more potent than that obtained from heart (compare FIGS. 2 and 4). Since cardiophilin from both liver and heart contain linoleic acid as the predominant fatty acid, it is likely that the isolated inhibitor from liver contains C-2-acyl, C-1-OH while the material synthesized enzymatically from heart cardiophilin is C-1-acyl, C-2-OH. Thus, structural isomerism is the probable cause for the difference in potency.

The method of this invention is useful both in treating a phospholipase A_2 mediated condition (PMC) or symptom which has already manifested itself in the mammal as well as the prevention of these conditions or symptoms in mammals particularly susceptible to them. Employment of the method of this invention prior to the development of a PMC would prevent the formation of the prostaglandins and similar products necessary for such conditions. Thus, the method of this invention can be used to prevent edema and erythema from sunburn by administering these compounds prior to exposure to sunlight. The compounds of this invention could be administered to persons suffering from hayfever or

similar allergies prior to exposure to allergenic substances which are particularly hard on hayfever sufferers. In a like manner, a physician or veterinarian could readily determine other mammals or persons susceptible to a PMC.

The actual inhibition of phospholipase A_2 by the method of this invention takes place on a cellular level. Administration of the compound of this invention can thus be by any manner which will allow for phospholipase A_2 inhibition in the affected tissues or organs. The preferred route in most cases would be to systemically administer the compounds, i.e., to allow them to enter the mammal's bloodstream and thus be administered throughout the mammal's system. In certain cases, where the PMC is of a localized nature (e.g., sunburn), topical administration (e.g., transdermal) may be employed in order that the phospholipase A_2 inhibition is confined to the afflicted area.

Methods of administering these compounds will depend on the particular phospholipase mediated condition (PMC) sought to be treated. Regardless of the route of administration selected, the compounds used in the method of the present invention are formulated into pharmaceutically acceptable dosage forms by conventional methods known to the pharmaceutical art.

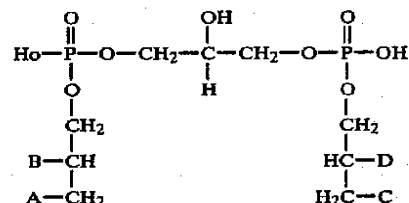
They may also be administered buccally, rectally or vaginally in forms such as suppositories. They may also be introduced parenterally, subcutaneously, or intramuscularly using sterile injectable forms known to the pharmaceutical art.

The dosage regimen for preventing or treating phospholipase mediated conditions (PMC) by the compounds of this invention is selected in accordance with a variety of factors, including the type, age, weight, sex and medical condition of the mammal, the severity of the PMC and the particular compound employed. An ordinarily skilled physician or veterinarian will readily determine and prescribe the effective amount of the anti-PMC agent to prevent or arrest the progress of the condition. In so proceeding the physician or veterinarian could employ relatively low dosages at first, subsequently increasing the dose until a maximum response is obtained.

The invention has been described with respect to various specific and preferred embodiments. However, it should be understood that many variations or modifications may be made while remaining within the spirit and scope of the invention.

What is claimed is:

1. A method for *in vivo* inhibition of phospholipase A_2 comprising administering to a mammal an effective amount of a compound of the formula;



wherein A is R_1CO_2 OH, B is R_2CO_2 or OH, D is R_3CO_2 or OH and C is R_4CO_2 ; wherein R_1 , R_2 , R_3 and R_4 are each $C_{10}-C_{30}$ alkyl groups comprising from about 0-3 double bonds; and wherein at least A, B or D is OH.

2. The method of claim 1 wherein each of R_1 , R_2 , R_3 and R_4 is a C_{12} - C_{22} alkyl group.

3. The method of claim 1 wherein B is OH, A is R_1CO_2 and D is R_3CO_2 .

4. The method of claim 3 wherein the compound is monolysocardioliipin.

5. The method of claim 1 wherein A is OH, B is R_2CO_2 and D is R_3CO_2 .

6. The method of claim 5 wherein the compound is isomono-lysocardioliipin.

7. The method of claim 1 wherein B is OH, A is R_1CO_2 and D is OH.

8. The method of claim 7 wherein the compound is dilysocardioliipin.

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